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Bioassay and Preliminary Characterization of Ovigerous-Hair Stripping Substance (OHSS) in Hatch Water of Crab Larvae

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Abstract. Hatch water (the filtrated medium into which zoea larvae have been released) of the estuarine terrestrial crab *Sesarma haematocheir* (akate-gani) contains a substance that causes premature detachment of embryos from ovigerous females. Detachment occurs when the ovigerous hairs along the female's ovigerous setae slip out of the investment coat that binds them to the embryos through stalks, or funiculi. The active factor, which I call ovigerous-hair stripping substance (OHSS), is released outside of the egg capsule at the time of hatching, and is not secreted by the female. This study describes the results of a quantitative assay for measuring the activity of OHSS. Activity is measured as the percentage of hairs on a seta that can be induced to slip out of the coat without damage. Experiments with an extract of crushed embryos indicated that OHSS is present up to 2 days before hatching. Its activity was destroyed by heat and trypsin, suggesting that it is a protein. Its molecular size was estimated by gel filtration to be 15–20 kDa in *S. haematocheir* and 30 kDa in *S. pictum*. Reciprocal tests among different species indicated that OHSS occurs widely in intertidal and estuarine crabs.

Introduction

After oviposition, the embryos of decapod crustaceans are wrapped in a thick membrane and clustered on the ovigerous setae beneath the abdomen of the female. Many fine hairs (*i.e.*, ovigerous hairs) are arranged along the seta, and the embryos are attached to these hairs by a stalk: the funiculus. The mechanism by which the fertilized egg is attached to the ovigerous hairs and the source of the material that makes up the funiculus and the egg capsule have been subjects of controversy for many years (*e.g.*, Andrews, 1906; Yonge, 1937, 1946; Mawson and Yonge, 1938; Linder, 1960; Suko, 1961; Cheung, 1966; Fisher and Clark, 1983; Goudeau and Lachaise, 1980, 1983; Goudeau *et al.*, 1987; Talbot and Demers, 1993).

In addition to the funiculus, the embryo attachment system involves a clear coat that wraps around (invests) the ovigerous hairs (Saigusa, 1994). The funiculus is therefore not connected to the ovigerous hairs directly, but indirectly through the coat—a fact not previously reported. The investing coat may be composed of the same materials that make up the funiculus and the outer layer of the egg capsule, but this notion has not been tested adequately.

While attached to the ovigerous hairs, the embryos are ventilated by the movement of pleopod setae. When development is completed, the egg capsule breaks, and the zoeas hatch and are released into the water by a special fanning movement of the female's abdomen (larval release is described in Saigusa, 1982). After the larvae have been released, the empty egg cases, funiculi, and investing coats remain attached to the ovigerous hairs. Soon, however, a substance released at the time of hatching causes the ovigerous hairs to slip out of the investment coat, detaching the funiculi and empty egg cases; the active factor is called 'ovigerous-hair stripping substance' (OHSS) (Saigusa, 1994). Within a few hours, the ovigerous hairs are cleaned without damage, and in a few days a new clutch of fertilized eggs is attached to the hairs. Because OHSS is released at the time of hatching, one might suppose it to be directly involved in hatching. But there is no evidence that this factor is a hatching enzyme of the sort known in many groups of animals (Saigusa, 1994).

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If the medium in which hatching and larval release has occurred is filtered, the resulting solution—called hatch water—can affect other ovigerous females, causing all of their embryos to slip off the ovigerous hairs and out of the brooding space without hatching. This effect on whole crabs was used in the preceding experiments (Saigusa, 1994) to assay the activity of the OHSS contained in hatch water. To investigate the properties of this substance further, however, an assay that would require only small amounts of active material was essential.

In this study, therefore, I have used only segments of ovigerous setae with their attached embryos in a quantitative assay of the effect of OHSS. This substance is a protein released into the medium at the time that the egg capsule breaks. The molecular weight of OHSS was estimated by gel filtration. Reciprocal tests with several species suggest that OHSS occurs widely in intertidal and estuarine crabs.

Materials and Methods

Handling of ovigerous females for assays

Individuals of the terrestrial red-handed crab, *Sesarma haematocheir,* were collected from the thicket along a small estuary at Kasaoka, Okayama Prefecture, Japan, in 1992 and 1993. After collection, the crabs were quickly brought into the experimental rooms, where they were kept in several plastic containers (70 cm long, 40 cm wide, and 25 cm high) containing shallow water (about 1 cm deep) and hiding spaces. The light and temperature were controlled, respectively, at LD 15:9 and 24 ± 1°C. The assay is sensitive and seemed to be influenced by even very small quantities of OHSS mixed into the water. So when one or more females released their larvae into a container, *all* the females kept in that container were returned to the field. To avoid this problem, females were usually used for assay within a few days after collection.

Preparation of hatch water

As reported elsewhere (Saigusa, 1994), OHSS is contained in hatch water, *i.e.,* the filtered medium in which zoea larvae have hatched. The color of the embryos carried by females changes from dark brown to brownish green according to the stage of development, which can, therefore, be estimated by visual inspection. To obtain hatch water, females with mature embryos (brownish green) that were due to hatch within a few days were collected in the field (*i.e.,* in Kasaoka) (Saigusa, 1982).

As shown earlier (*e.g.,* Saigusa, 1988), hatching and larval release by estuarine crabs are under the control of a circatidal rhythm the phase of which can be shifted in the laboratory by the 24-h light cycle. When these females were kept under a day-night cycle in phase with that in the field (*i.e.,* light-on at 0500 and light-off at 2000), the larvae hatched at night at about the time of high tide. The ovigerous females were placed individually in glass or plastic beakers (8.5 cm in diameter, 12 cm in height) containing 30 ml of diluted seawater (10 ppt) or the same quantity of distilled water. The solution was aerated for 1 day before use. The medium was changed every day if larval were not released.

Hatching of estuarine crabs is highly synchronized; all of the embryos may hatch within about 5–30 min in the laboratory (see Saigusa, 1992, 1993). As soon as hatching was complete and the female had released all of her zoeas into the medium, she was removed, and the medium was filtered through nylon mesh to remove the zoeas. This filtered medium was immediately transferred to a small bottle, and was stored at −15°C until used; at this temperature, the activity of OHSS is maintained for at least half a year. But almost all of the experiments were done with hatch water that had been collected within the month.

Assay of OHSS activity

I have not yet found an artificial substrate that is acted upon by OHSS, so an efficient biological assay was developed, as follows. Female crabs have four pairs of abdominal appendages, each of which consists of plumose and ovigerous seta. Embryos are attached to the ovigerous hairs arranged along the ovigerous seta, by the funiculi (for details, see Saigusa, 1994). Several ovigerous setae with their clusters of embryos still attached were cut off from many females and subdivided, usually into six segments, under the stereomicroscope (Fig. 1a, b). Each segment with its cluster of embryos—hereinafter called an egg cluster—(Fig. 1a) was immersed in 0.5 ml of 10 ppt seawater (SW), or the same quantity of hatch water. The medium and egg clusters were incubated for various times in a plastic culture dish with 24 wells, each 1.6 cm in diameter and 1.7 cm in height. This dish was shaken back and forth (3–4 cm) at 100–120 times per min in the experimental rooms.

After the incubation, each egg cluster was again placed in a glass dish with 10 ppt SW or distilled water. The dish was put under a stereomicroscope, and fine forceps were used to pull the embryos gently away from their seta. When an egg cluster incubated in 10 ppt SW was pulled, about 90% of the ovigerous hairs were broken away from the seta (Fig. 1c). But of the clusters incubated with hatch water, about 80% slipped off easily and without damage (Fig. 1d). The activity of OHSS was therefore taken as the percentage of the hairs stripped clean and undamaged to the total number of hairs along the segment of seta.

Time course of the effect of OHSS with diluted hatch water

Hatch water collected from a single female was diluted 3, 9, and 27 times, and the time course of OHSS activity

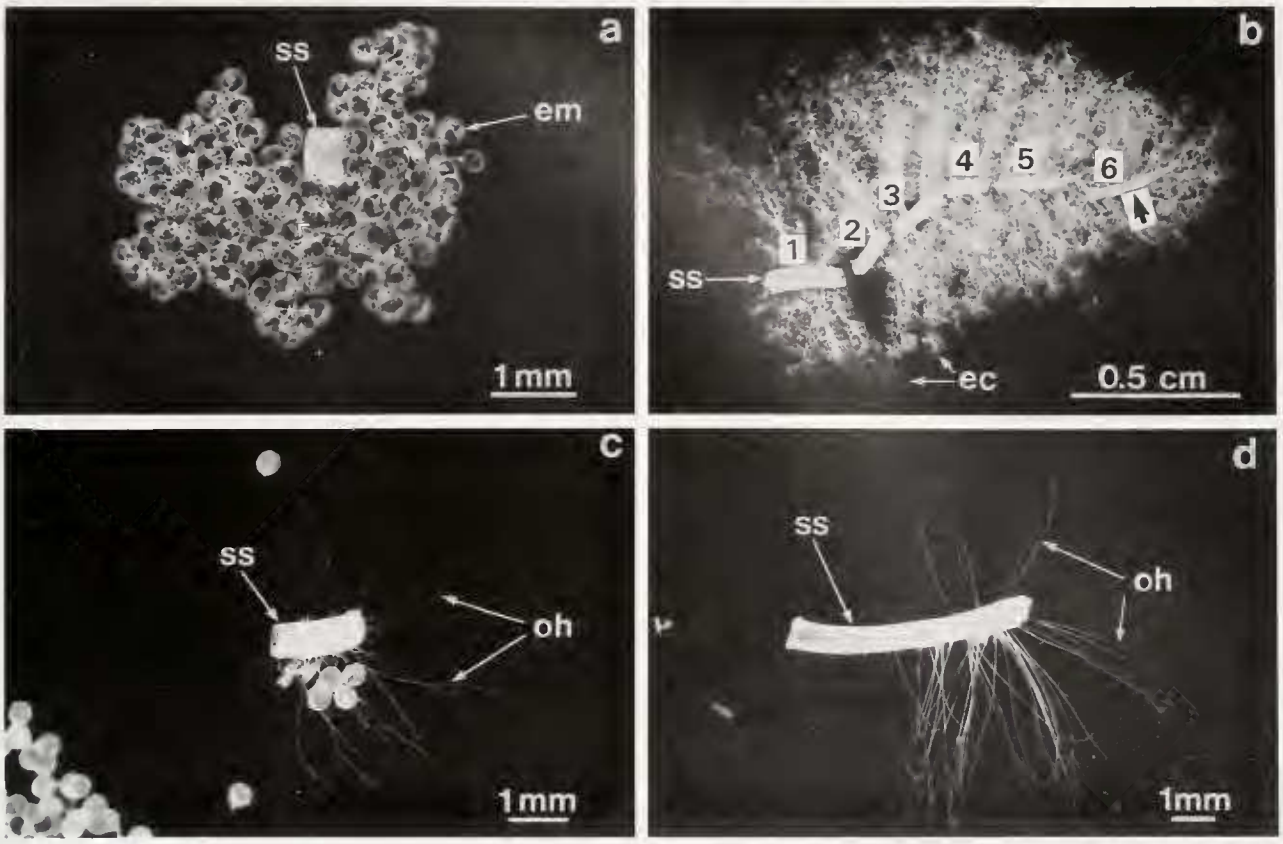

Figure 1. A method for assessing the activity of OHSS. (a) One of the setal segments (*ss*) subdivided before hatching, with its developing embryos (*em*) still attached. (b) An ovigerous seta is cut into six pieces (1–6). The arrow shows where the tip of the seta is removed (see text for details). This figure shows a seta just after the zoeas have hatched, leaving the empty egg cases (*ec*) still attached to ovigerous hairs which, however, are visible. (c) Ovigerous hairs (*oh*) incubated in 10 ppt SW, and the attached embryos then pulled gently away with forceps. (d) The same, but the ovigerous hairs (*oh*) incubated in hatch water. Note that many of the hairs incubated in seawater are broken off (compare c and d).

was compared with that of undiluted samples. This experiment was carried out with hatch water of *S. haematocheir* and *S. pictum*.

Appearance of OHSS activity in living females

To determine when OHSS is released, either by the larvae or by the female, one or two ovigerous setae were detached from the female before and after hatching. Before hatching, unhatched embryos were attached to the setae, but after the release of larvae, only the remnants (*i.e.*, broken egg capsules, funiculi, and the investment coat) remained on the setae. These setae were subdivided, and the embryos or the remnants were pulled with forceps. Time of hatching and larval release was determined and recorded by the photoelectric switch method described earlier (Saigusa, 1992, 1993).

Presence of OHSS activity before hatching

Embryonic development of *S. haematocheir* can be divided into four stages by visual inspection: the early stage (10 days), from the start of cleavage to just before the formation of eye pigments; the intermediate stage (10 days), from eye pigment formation to completion of the compound eyes; the late stage (1 week), in which the egg color changes from brown to brownish green; and the final stage (*i.e.*, mature embryos), in which hatching should occur within a few days.

To examine whether active OHSS is present before hatching, egg clusters of various developmental stages were examined: *i.e.*, two or three ovigerous setae with their attached embryos were detached from the females and crushed by hand for a few minutes, with 3 ml of 10 ppt SW per one seta. Aliquots (0.5 ml) of this embryo extract, uncentrifuged, were immediately pipetted into the wells of a culture dish. Freshly detached egg clusters were placed into this solution, and OHSS activity was monitored 4 h later. Time of hatching and larval release by the females that had yielded the experimental egg clusters was also monitored by the photoelectric switch method.

Susceptibility of OHSS to trypsin

Two milligrams of trypsin (porcine pancreatic "trypsin 1:250," Difco Laboratories) was dissolved in 20 ml of hatch water. The solution was divided into aliquots and incubated at 35°C for either 75 min or 3 h. These solutions were then transferred to room temperature (about 25°C). Egg clusters were placed into this solution, and OHSS activity was examined for the next 4 h. Furthermore, to test whether trypsin itself causes the wrapping coat to slip off the ovigerous hair, egg clusters were incubated for 4 h at 25°C, with 0.5 ml of 10 ppt SW containing only trypsin, and at the same concentration.

Gel filtration

Hatch water collected from several females was centrifuged at 15,000 rpm for 30 min at 5°C to remove the solid materials. The supernatant was freeze-dried and was then reconstituted in 1 ml of 10 m*M* Tris-HCl buffer (pH 7.5). This test sample, containing Blue Dextran (Pharmacia) and 1 M NaCl for calibration, was applied to a Sephacryl S-200 (Pharmacia) column (45 cm × 1.3 cm i.d.), and fractions (0.8 ml) were collected at 10-min intervals. The column was eluted with the Tris-HCl buffer, and the protein in each fraction was monitored with a Beckman DU-65 spectrophotometer at O.D. 280 nm.

The activity of OHSS in the fractions from gel filtration was determined by the method of Shirai (1986), as follows. A series of threefold dilutions of each active fraction was prepared, and an egg cluster was immersed in each dilution and tested by gentle pulling with forceps. The response—the percentage of stripped, undamaged hairs in each solution—was then plotted against the log of the dilution (Fig. 2). The potency of a fraction was expressed as the dilution producing a half-maximal effect (ED_{50}). But because the maximal response in this assay is about 80% and the minimum is about 10% (dashed lines in Fig. 2), the ED_{50} was taken as the dilution producing 45% stripped ovigerous hairs (shown as –· ··–· ·– in Fig. 2).

OHSS in other species. Females of *S. erythrodactylum*, *S. pictum*, and *S. dehaani* bearing embryos that appeared likely to hatch within a few days were collected from the field at Kasaoka and brought into the laboratory. Hatch water from these crabs was obtained in the same way as from *S. haematocheir*, although the quantity and salinity of the medium varied with the body size of each species and the ambient water into which larvae would normally be released (*i.e.*, 20 ml of 20 ppt SW for *S. pictum;* 15 ml of 20 ppt SW for *S. erythrodactylum;* and 40 ml of 10 ppt SW for *S. dehaani*). The filtered medium was frozen at −15°C until it was used.

Hatch waters of these additional three species and *S. haematocheir* were applied to the unhatched embryos of six species (listed in Table II). Females carrying unhatched embryos were collected from each habitat, and the ovigerous setae with their attached egg clusters were cut into 2–6 pieces according to the size of the crab. Experimental procedures were the same as for *S. haematocheir*. Time of incubation was 4–6 h, depending upon the species that provided the egg clusters.

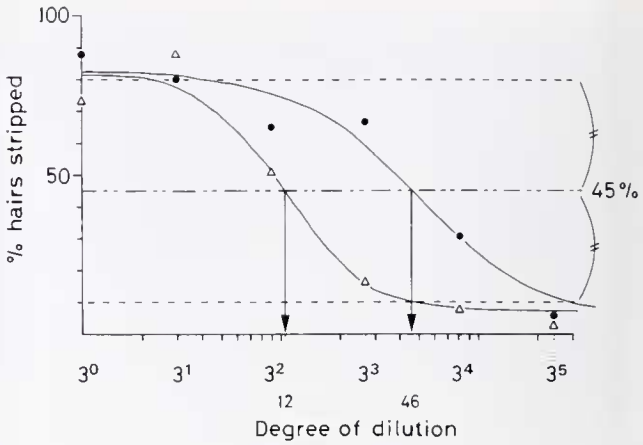

Figure 2. Dilution-response curves of crude and concentrated hatch water; the degree of dilution is scaled logarithmically (base 3). Broken lines show the maximum and minimum percentages of ovigerous hairs that were stripped off the investing coat without damage. The dilution-response curves for crude hatch water (open triangles) and concentrated hatch water (black circles) were drawn by hand without statistical treatment. Activity units are taken as the values of the dilution at which each dose-response curve intersects the 45% level (downward arrows): *i.e.*, 12 (crude) and 46 (concentrated) units in these preparations.

To examine further whether the effect of OHSS is different among species, the hatch water collected from several females of *S. pictum* was pooled, and assayed with egg clusters of *S. haematocheir, S. erythrodactylum,* and *Hemigrapsus sanguineus.* The time course of the effect of OHSS was monitored every 15 min or 1 h.

Results

Development of the assay

The following preliminary experiments were aimed at understanding the variables in the assay and thus improving its reliability. The ovigerous setae with their egg clusters were cut off and subdivided, usually into six segments (Fig. 1a). As shown earlier (Saigusa, 1994), most ovigerous hairs are arranged in whorls along the ovigerous seta. The length of these hairs depends upon their position along the seta (Fig. 1b): *i.e.*, the hairs along positions 2–5 are longest; those at the tip of the seta (position 6) are shortest; and those at the base (position 1) are of intermediate length (see also Fig. 6B in Saigusa, 1994).

The first question was whether the length of the hairs would affect the ease with which they slip out of the investing coat. When incubated with 10 ppt SW, the shortest

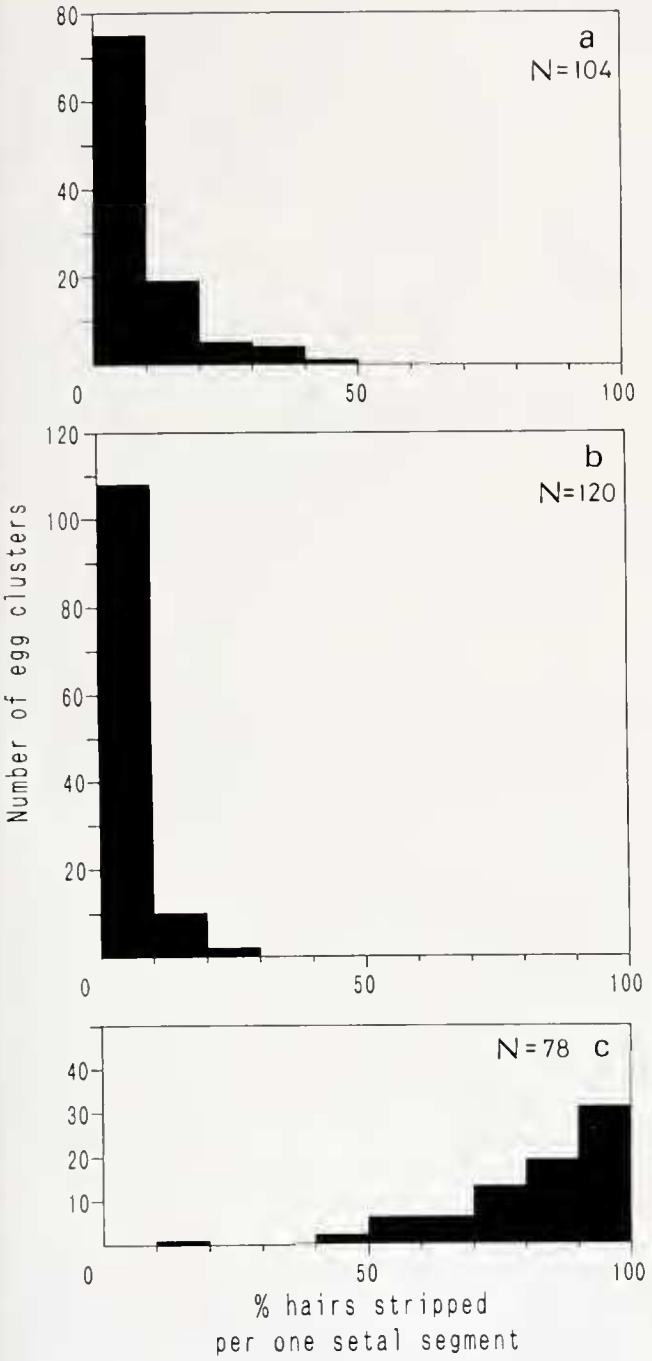

Figure 3. Distribution of the number of segments in which the percentage of the ovigerous hairs that slipped out of the coat without damage was estimated. (a) Data from experiments in which the tip of the seta remained (incubation in 10 ppt SW). (b) Data from experiments in which the seta was cut at its tip as shown in Figure 1a (incubation with 10 ppt SW). (c) Embryo clusters treated with hatch water. N = Total number of subdivided setal segments with their attached egg clusters. The percentage for each setal segment is shown at intervals of 10%.

hairs certainly tended to slip out most easily. This tendency was especially marked at the tip of the seta (Fig. 3a). So in most of the experiments, the tip of the ovigerous seta was cut away before use (arrow in Fig. 1b). On average, only 10% of the hairs were stripped clean without damage (Fig. 3b). In contrast, about 80% of the egg clusters slipped off when the preparation was placed into hatch water (Fig. 3c).

A further difficulty is that the response to a given concentration of OHSS is considerably different even among the egg clusters produced by the same female. Figure 4 shows the response of two groups of the egg clusters that were separated from one female and both immersed in hatch water with the same concentration of OHSS. Although the percentage of stripped hairs fluctuated considerably, it clearly increased with time, reaching a maximum in 1–3 h (*e.g.*, Fig. 4). In contrast, control egg clusters immersed in 10% seawater showed no such increase in the percentage of stripping. The fluctuation in the responses suggests that the adhesion between the investing coat and the ovigerous hairs is also variable, even in the same female.

Concentration-dependence of OHSS activity

Unhatched embryos detached from two females of *S. haematocheir* were incubated with a series of threefold dilutions of hatch water collected from one female, and the time course of the effect of OHSS was monitored (Fig.

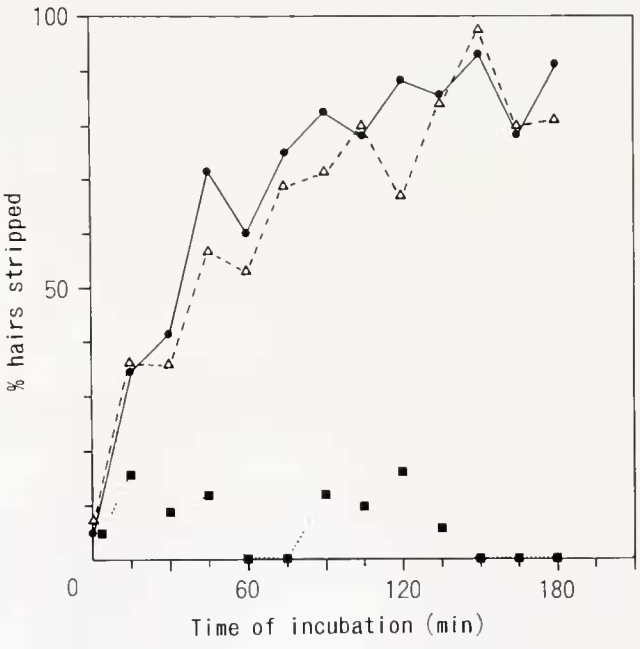

Figure 4. A typical time course of the effect of *S. haematocheir* hatch water on the stripping of unhatched embryos of the same specimen. Two egg clusters were incubated in hatch water from one female (●—● and △- - -△), and one incubated in 10 ppt SW (■· · ·■). These egg clusters were all separated from a single female. The broken line shows the response curve of the mean value between the two preparations immersed in hatch water.

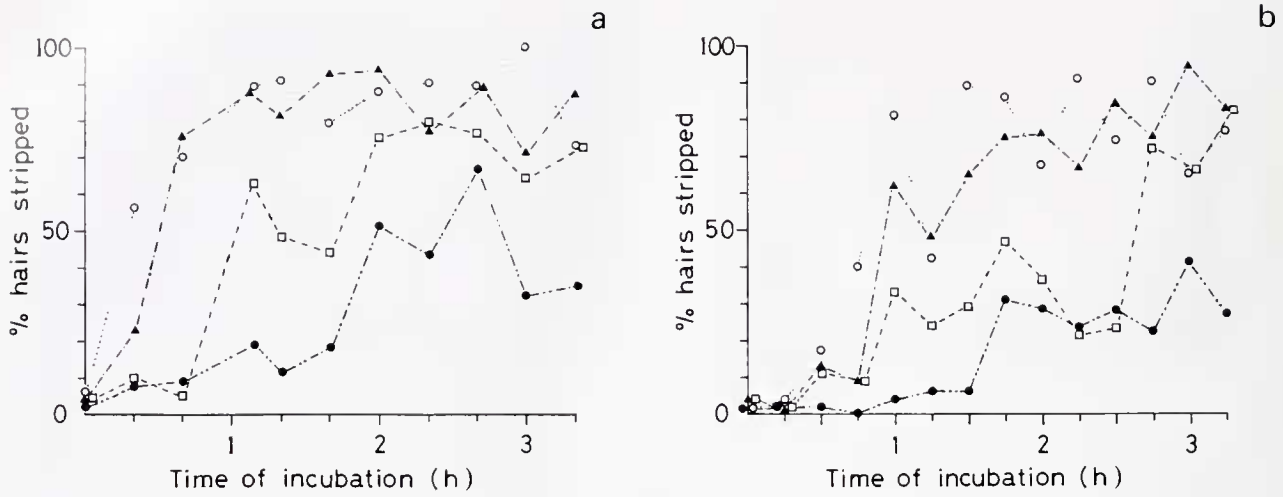

Figure 5. Time course showing the effect of diluting of hatch water with 10 ppt SW. (a) *S. haematocheir* embryos incubated with *S. haematocheir* hatch water. (b) *S. pictum* embryos incubated with *S. pictum* hatch water. No dilution (○· · ·○); 3-fold dilution (▲–·–·–▲); 9-fold dilution (□- - -□); 27-fold dilution (●–· · –●).

5a). In the groups of embryos placed into undiluted and threefold-dilute hatch water, half-maximal activity of OHSS was reached in 20–30 min and was largely completed (about 85%) after 60–80 min of treatment. A ninefold dilution of hatch water became equiactive to the undiluted solution in an hour, and the maximum response was decreased to 60%–70%. Finally, the maximum activity of a 27-fold dilute solution was reduced about 30%.

The effect of dilution of hatch water was also tested in *S. pictum* (Fig. 5b). In this experiment, embryos detached from two females of *S. pictum* were placed into a series of threefold dilutions of *S. pictum* hatch water. Although the percentage of slipped hairs also fluctuated in this species, the trends in Figure 5b were very similar to those of Figure 5a.

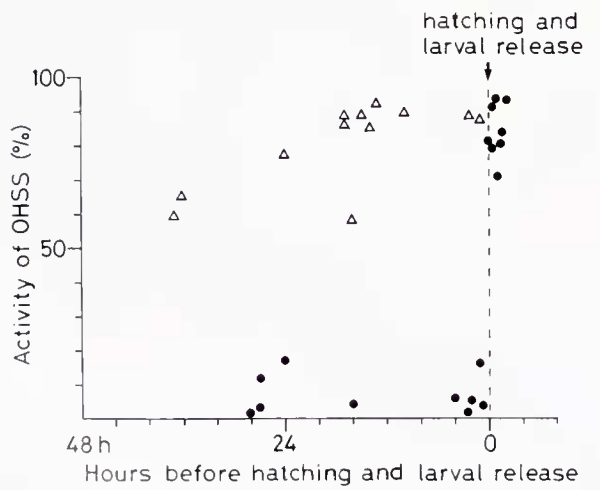

Figure 6. OHSS activity within the embryos before, and just after, hatching and larval release. Solid circles (●): percentage of stripped hairs on setae detached from the females. These setae were taken from females with unhatched embryos (before hatching and larval release), as well as those with remnants (after the release of larvae). They are not treated with hatch water. Open triangles (△): hairs on setae that were incubated in a solution of crushed embryos for 4 h, and then pulled with forceps.

Appearance of the activity in the embryo and the timing of release outside the egg membrane

To determine the time that OHSS has an effect on living females, the activity of the substance before the time of hatching and larval release was compared with the activity afterward. Until just before hatching, most ovigerous hairs were broken when the egg cluster was detached and pulled with a forceps; but just after larval release, the hairs easily slipped out of the coat (solid circles in Fig. 6). This indicates that OHSS is released at the time of hatching, either by the zoeas or by the female.

Furthermore, to determine whether the OHSS activity appears in the embryos before hatching, freshly detached embryos were incubated with the material containing crushed embryos. No activity was detected in the embryos, even at late stages of development (data not shown). But activity was clearly detected in embryos that should have hatched within 48 h (triangles in Fig. 6). Thus, OHSS seems to accumulate in the embryos before hatching. Because the activity is not detected outside of the egg case just before hatching, we can suppose that its release is associated with breakage of the egg capsule.

Gel filtration

Lyophilized hatch water collected from four specimens of *S. haematocheir* was redissolved and subjected to gel

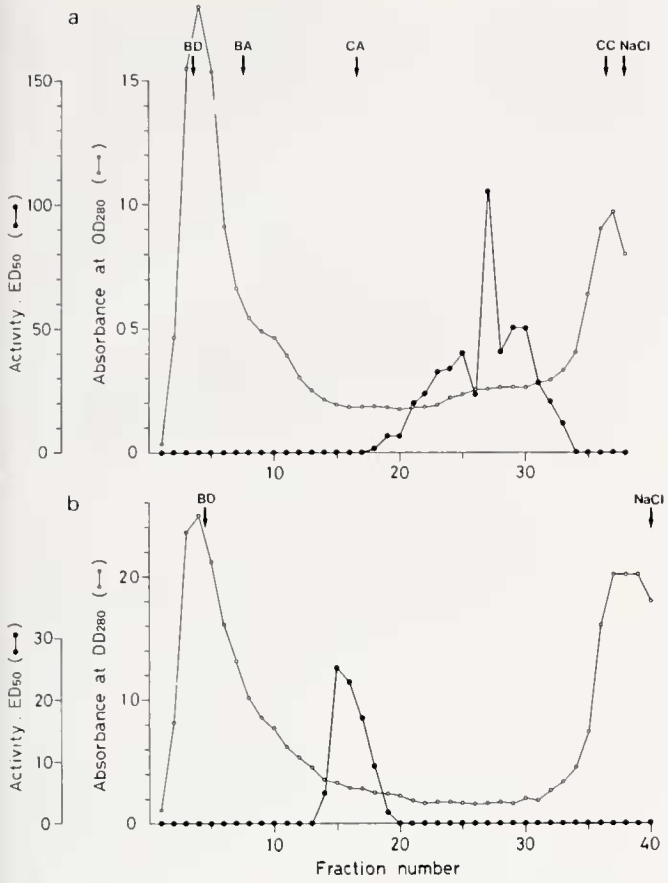

Figure 7. Gel filtration of hatch water. (a) *S. haematocheir.* Lyophilized hatch water collected from four specimens was reconstituted and passed through a Sephacryl S-200 column equilibrated with 10 m*M* Tris-HCl buffer (pH 7.5). Egg clusters of *S. haematocheir* were incubated with each fraction for 1.5 h, and the OHSS activity was assayed (protocol in text). Downward arrows show elution peaks of blue dextran (BD), bovine serum albumin (BA), carbonic anhydrase (CA), cytochrome c (CC), and NaCl. These markers were passed through the column without hatch water. (b) *S. pictum.* Test sample was obtained from three females of *S. pictum,* and passed through the same column after reconstitution with the same buffer. Embryos of *S. haematocheir* were incubated for 5 h, and the OHSS activity was then assayed. Note species differences.

filtration on a column of Sephacryl S-200. The activity of each fraction was assayed with unhatched embryos of the same species. As shown elsewhere (*e.g.,* Fig. 5), activity is variable, so the experiments were repeated 12 times, always with similar results. Figure 7a shows one of these experiments. The activity of OHSS extends over a wide range of fractions. The molecular size of OHSS was estimated to be 15–20 kDa by a comparison of its elution volume with those of standard proteins: *i.e.,* bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa).

Hatch water collected from three females of *S. pictum* was subjected to the same gel filtration protocol that was used with *S. haematocheir,* and the activity was also assayed with embryos of *S. haematocheir.* This separation was repeated four times with similar results. As shown in Figure 7b, OHSS of *S. pictum* eluted as a single peak, and the molecular size was estimated by comparison with standards to be about 30 kDa.

Table I

The effect of boiling and trypsin treatment on the OHSS activity of hatch water from Sesarma haematocheir

Experiments	Percentage of stripped hairs ($\bar{X}$ ± SD)*
Dilute SW (10 ppt)	0 (±0)
Trypsin solution	2.6 (±4.7)
Hatch water	82.2 (±9.8)
Boiling of hatch water	3.6 (±2.5)
Trypsin treatment of hatch water (75 min)	69.1 (±11.8)
Trypsin treatment of hatch water (4 h)	8.3 (±6.8)

* In all cases, the number of setal segments tested was 12.

Effect of trypsin

A few tests were conducted to examine the nature of OHSS. As indicated in Table I, its activity was eliminated when hatch water was heated for 5 min. The activity was also somewhat decreased when the hatch water was treated with trypsin for 75 min, and was completely lost with 3 h of treatment. On the other hand, trypsin itself did not affect the coat, although this enzyme often decomposed the basement of the ovigerous hairs. These experiments suggest that OHSS is a protein.

Species distribution of OHSS activity

Table II summarizes the reciprocal tests in which hatch waters from four species of *Sesarma* were applied to the

Table II

Reciprocal tests on the effect of OHSS

Source of hatch water	Source of cluster of embryos	Percentage of stripped hairs ($\bar{X}$ ± SD)*	
		Hatch water	10 ppt SW
Sesarma dehaani	*S. haematocheir*	81.9 ± 9.2 (12)	3.8 ± 4.1 (12)
S. erythrodactylum	*S. erythrodactylum*	84.5 ± 13.1 (6)	5.2 ± 5.7 (6)
	S. pictum	89.3 ± 6.3 (4)	2.2 ± 3.8 (4)
	S. haematocheir	61.7 ± 18.6 (6)	3.7 ± 5.1 (6)
S. haematocheir	*S. bidens*	99.2 ± 1.3 (4)	8.4 ± 5.5 (4)
	Gaetice depressus	49.7 ± 8.9 (6)	1.9 ± 2.1 (6)
S. pictum	*Hemigrapsus sanguineus*	41.7 ± 22.5 (6)	1.6 ± 1.0 (6)
	S. erythrodactylum	94.1 ± 3.7 (6)	3.7 ± 3.5 (6)
	S. haematocheir	60.8 ± 12.1 (4)	2.6 ± 2.6 (4)
	S. pictum	64.1 ± 11.5 (6)	5.0 ± 6.3 (6)

* Number of setal segments tested is in parentheses.

embryos of six species of crab. All species were affected by all types of hatch water, suggesting that OHSS occurs widely in intertidal and estuarine crabs.

But there was a clear difference in the time course of activity among species. Figure 8a, b summarizes the results of experiments in which *S. pictum* hatch water with the same OHSS concentration was applied to the embryos of *S. erythrodactylum*, *S. haematocheir*, and *Hemigrapsus sanguineus*. In *S. erythrodactylum*, the maximal activity was reached after 1 h of incubation (Fig. 8a), but more than 5 h was needed for *H. sanguineus* (Fig. 8b).

Discussion

Hatch water of the estuarine terrestrial crab *Sesarma haematocheir* contains a substance that causes premature detachment of embryos from ovigerous females. The active factor—ovigerous-hair stripping substance (OHSS)—is released outside of the egg case at the time of hatching, and is not released by the female. Its molecular size was estimated by gel filtration to be 15–20 kDa in *S. haematocheir* and 30 kDa in *S. pictum*. These results raise the following five issues in relation to the OHSS activity and its function: species specificity, timing of synthesis and secretion, mode of action, characterization, and composition of the investment.

Species specificity of OHSS activity

As shown in Figure 7b, OHSS activity of *S. haematocheir* can be assayed with egg clusters of *S. pictum*. Reciprocal tests among different species indicate that OHSS occurs widely in intertidal and estuarine crabs (Table II). Thus, the hatch water from one species of crab has an effect on the embryos of other species. On the other hand, a given concentration of hatch water from one species can, in its action on crabs of other species, have very different time courses (Fig. 8). This disparity suggests that the response to OHSS differs among species. For example, the length of the ovigerous hairs differs among species, so the strength of the bond between the coat and the ovigerous hairs might also differ, causing the variation in time course. Moreover, the molecular size of OHSS is different in *S. haematocheir* and *S. pictum* (Fig. 7). This suggests that the molecular structure of OHSS might also vary, and it might have a different effect when applied to the embryos of other species.

Timing of OHSS synthesis and secretion

As shown elsewhere (Saigusa, 1992), clusters of *S. haematocheir* embryos that are detached from the female within 48–49.5 h of larval release all hatch successfully; but embryos that have been detached from the female for longer periods do not hatch at all, though they are obviously alive and have the potential of hatching (see also Saigusa, 1993). These results suggests that the hatching process is a distinct program initiated near the end of embryonic development (Saigusa, 1992).

No activity appeared outside of the egg case until hatching occurred. But a homogenate of embryos clearly showed that the activity was present in embryos that should have been in the process of hatching (Fig. 6). Because no activity was detected in earlier developmental

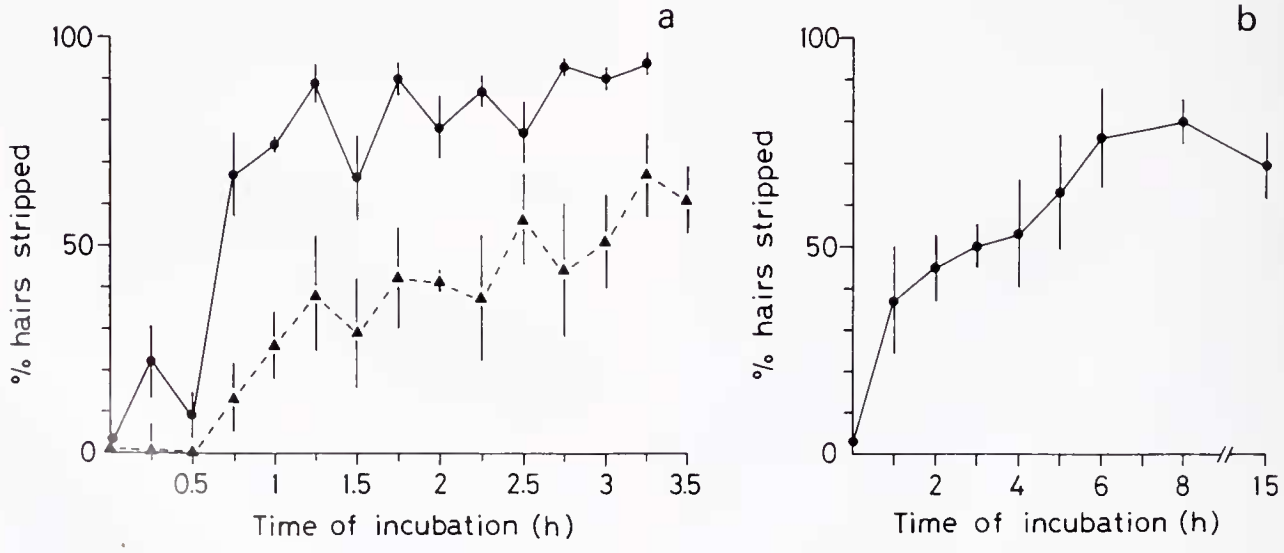

Figure 8. Time course of the effect of OHSS when hatch water from *S. pictum* was applied to the embryos of other species. (a) Embryos of *S. erythrodactylum* (solid circles) and *S. haematocheir* (solid triangles); (b) Embryos of *Hemigrapsus sanguineus* (solid circles).

stages, we can speculate that OHSS begins to be produced only immediately before the start of hatching. Further studies are required to determine the exact timing of OHSS synthesis and secretion in the unhatched embryos.

Mode of action of OHSS

The embryos that slip off the ovigerous hairs are always associated with the funiculus and the wrapping coat, and scanning electron microscopy revealed no morphological changes, either on the coat or the hair (Saigusa, 1994). Goudeau and Lachaise (1983) showed a cross section of the wrapping coat in the shore crab *Carcinus maenas*. Their figure suggests that the material of the funiculus is coiled around the ovigerous hair without any additional substance. If this is the case in *S. haematocheir*, it is not plausible that OHSS invades the space between the coat and the ovigerous hair. So I suppose that OHSS works directly on the coat, perhaps softening it, so that the hairs can separate from the investment coat. But what is the mechanism by which this substance softens the coat?

Identity of OHSS

One possibility is that OHSS is a protease that partially digests the layers of the investment coat. Embryos of many groups of animals (*e.g.*, fishes, sea urchins, and amphibians) release a protease that digests egg membrane or egg capsule to cause hatching (for a review, see Saigusa, 1994). DeVries and Forward (1991) also reported a proteolytic enzyme released near the time of hatching in three species of estuarine crabs. It is not known whether OHSS is a proteolytic enzyme, nor whether it acts as a hatching enzyme. But if so, then OHSS may partially degrade, not only the egg capsule, but also the investment coat, causing them to slip off the ovigerous hairs.

Another possibility is that OHSS is a type of chitinase such as is known in many kinds of animals and plants. Chitin is a stable sugar complex constituting the exocuticle, endocuticle, and membraneous layer in crustaceans. It is hydrolyzed by two enzymes in sequence: chitinase, which converts the long chitin polymers into small oligosaccharides; and chitobiase (β-*N*-acetylglucosaminidase), which hydrolyzes these chito-oligomers into *N*-acetyl-D-glucosamine (Lunt and Kent, 1960; Jeuniaux, 1966; O'Brien *et al.*, 1993). These enzymes might be degrading the coat wrapping the ovigerous hairs until it is able to slip off the hairs. The problem with this notion is that such enzymes might also degrade the skin of the zocas.

Composition of the investment coat, funiculus, and egg envelope

In crabs, the funiculus extends to the coat investing the ovigerous hair, which suggests that the same material forms both the funiculus and the investment coat (Saigusa, 1994). As shown in Goudeau and Lachaise (1980), the structure and formation of the egg envelope of the shore crab *Carcinus maenas* are complex. Cheung (1966) showed that the egg envelope and funiculus of this species are not affected by pepsin or trypsin, though the yolk of the embryos is easily digested. Cheung concluded that at least the outer layer (*i.e.*, trichromatic membranes in her paper) consists largely of non-proteinous substances. Furthermore, our pictures by the transmission electron microscopy indicate that this material makes up the surface layer of the egg envelope of crabs (unpub. data). If OHSS softens the tissue of the investment coat, it would also work on the outer layer of the egg envelope, softening the envelope. This might contribute to the case of hatching and might enhance hatching synchrony. In any event, morphological and experimental studies on this coat might help in identifying OHSS.

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